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(57) Abstract

A composition which, upon reacting with thrombin, functions as a fibrin sealant and is characterized as devoid of infectious agents such as bacteria or viruses and contains no protease inhibitors or other non-human proteins. Also, described is a method for producing the composition.

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TOPICAL FIBRINOGEN COMPLEX

This application is a continuation-in-part of U.S. Serial No. 755,156, filed September 5, 1991.

BACKGROUND OF THE INVENTION

Field of The Invention

The present invention relates to a fibrinogen composition and its method of preparation, wherein the composition can be used for wound closure in conjunction with thrombin and calcium.

Related Art

The attempt to use fibrinogen to achieve topical hemostasis was investigated as far back as the early 20th Century when fibrinogen patches for hemostasis were used in cerebral surgery. Later, mixtures of plasma and thrombin were used for skin grafting and intracavity injections in the therapy of tuberculosis. However, these early attempts had two major drawbacks: Since the source of fibrinogen was plasma, the concentration of fibrinogen was low which resulted in a fibrin film of insufficient strength; and it was not possible to inhibit the normal physiologic process of fibrinolysis such that the fibrin film was degraded relatively quickly.

Prior attempts to develop an effective fibrin sealant have also been hampered by the fact that most of these preparations contain high levels of plasminogen which required these compositions to additionally contain an anti-fibrinolytic agent in order to prevent premature degradation of the fibrin seal. Because anti-fibrinolytic agents are typically derived from a non-human source the possibility of a patient having an adverse reaction to such foreign proteins is significant, especially upon multiple exposure to these agents.

Although Rose, *et al.* (U.S. 4,627,879) report the production of a fibrin adhesive which does not necessarily require the presence of an anti-fibrinolytic additive, the composition disclosed in this reference does not deal with another major drawback of these prior fibrin sealing compositions, namely, the possible presence of infectious agents, such as Hepatitis B or HIV, in the plasma. As a consequence, the Rose reference requires that the compositions described therein be derived from a single donor, in order to avoid the transmission of infectious agents which might be associated with pooled plasma.

Thus, there is considerable need for a fibrin sealant which can be derived from pooled plasma and which is free of anti-fibrinolytic compounds, animal proteins, and infectious agents such as viruses. The present invention addresses these needs by providing such compositions.

SUMMARY OF THE INVENTION

The present invention is based upon the discovery that pooled plasma, even when substantially depleted of Factor VIII, could be processed to produce a fibrinogen preparation which reacts with thrombin and calcium, to produce a fibrin sealant that can be used to promote hemostasis.

In detail, the invention provides a fibrinogen composition which, in addition to being essentially free of Factor VIII and plasminogen, does not require the use of an anti-fibrinolytic agent and has been treated to eliminate the presence of infectious agents such as lipid enveloped viruses. A further advantage of the composition is that essentially all of the proteins present in the composition are human, i.e., encoded by a human DNA sequence.

The composition of the invention, through its transient *in vivo* presence, provides a matrix which persists for a period of time sufficient to achieve a medical effect, essentially lacks host toxicity upon degradation, and provides mechanical strength to promote hemostasis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 Schematic representation for preparation of Topical Fibrinogen Complex.

FIGURE 2 Effect of calcium ion concentration on fibrin polymer formation. Fibrinogen (90mg/ml) and thrombin (500U/ml) were mixed and allowed to incubate for 10 minutes. Lane A: MW markers; Lane B: Control fibrinogen; Lane C: 0mM Ca^{++}; Lane D: 1mM Ca^{++}; Lane E: 3mM Ca^{++}; Lane F: 6mM Ca^{++}: Lane G: 10mM Ca^{++}; Lane H: 20mM Ca^{++}; Lane I: 30mM Ca^{++}; Lane J: MW markers.

FIGURE 3 Effect of calcium ion concentration on fibrin polymer formation. Fibrinogen (130 mg/ml) and thrombin (500 U/ml) were mixed and allowed to incubate for 10 minutes. Lane A: MW markers; Lane B: Control fibrinogen; Lane C: 0 mM Ca^{++}; Lane D: 1mM Ca^{++}; Lane E: 3mM Ca^{++}; Lane F: 6mM Ca^{++}; Lane G: 10mM Ca^{++}; Lane H: 20mM Ca^{++}; Lane I: 30mM Ca^{++}; Lane J: MW markers.

FIGURE 4 Rate of fibrin polymerization. Fibrinogen (130 mg/ml) and thrombin (500 U/ml) were mixed in the presence of calcium ion (40mM $CaCl_2$). Lane A: MW markers; Lane B: 0 min; Lane C: 1 min; Lane D: 3 min; Lane E: 5 min; Lane F: 10 min; Lane G: 30 min; Lane H: 60 min; Lane I: 2 hr; Lane J: 4 hr; Lane K: 8 hr; Lane L: 24 hr; Lane M: fibrinogen and thrombin control; Lane N: MW markers.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The inventors have devised compositions for producing a fibrin sealant which represent a significant improvement over the prior art compositions intended to accomplish this effect. These compositions, termed Topical Fibrinogen Complex (TFC), are advantageous because they are able in the absence of non-human proteins and possible viral pathogens to achieve *in situ* longevity in order to induce hemostasis.

The compositions of the invention can be produced by starting with any blood-derived fraction which has not been significantly depleted of fibrinogen. Preferred blood fractions for producing the compositions of the invention are plasma, cryoprecipitate, and Factor VIII-depleted cold-precipitate. Generally, the process involves the formation of a cryoprecipitate which is high in Factor VIII (F VIII) and fibrinogen. This step is followed by a cold-precipitation which, in turn, contains a high concentration of fibrinogen and very low levels of F VIII. The addition of a calcium ion source during the cold-precipitation step enhances the precipitation of fibrinogen, as well as fibronectin, thereby increasing the concentration of these substances in the cold-precipitate. The addition of polyethylene glycol (PEG) can also be used to concentrate the fibrinogen in the composition.

More specifically, the method of the present invention typically starts with containers of frozen plasma. As described in Example 1, the cryoprecipitate is collected from the frozen plasma following thawing in a controlled environment.

Prior to formation of the cold-precipitate, the blood-fraction is treated to produce a cryoprecipitate. This cryoprecipitate can be produced by freezing the blood fraction (for example, plasma) which is thereafter warmed to a temperature not exceeding about +6°C. The cryoprecipitate is dissolved

in distilled water at about 20°C-35°C. Calcium chloride is added at a concentrate from about 1μM to about 1000 μM. Preferably, calcium chloride is added at this step at a concentration of about 40 μM and the pH adjusted to 6.8 ± 0.3 to enhance the precipitation of fibrinogen.

The dissolved cryoprecipitate is then cooled to 10°C ± 2°C with mixing, whereupon a precipitate, known as the cold-precipitate, forms. The precipitate is removed from solution by centrifugation, for example, 5600 g to 7200 g. The precipitate may be stored at -60°C or lower if desired. In the preferred embodiment, the initial fractionation to obtain the topical fibrinogen complex is accomplished by first performing a cryoprecipitation step followed by the cold-precipitation step.

The resulting precipitate, rich in fibrinogen, is dissolved in buffer with mixing and pH adjusted to 7.2 ± 0.1. Preferably the cold precipitate is resuspended in the presence of a protease inhibitor, such as PPACK (D-Phe-L-pro-L-arg-chloromethylketone), heparin cofactor II, or hirudin to inhibit thrombin which may be present. Most preferred as thrombin inhibitor is PPACK at a concentration from about 0.75 μM to about 1.75 μM. Thrombin inhibitor is removed in the PEG precipitation and DEAE column steps of the procedure. Those of skill in the art will know of other protease inhibitors of thrombin and their effective concentrations.

The cold precipitate suspension is transferred to a buffer solution containing a salt such as tri-calcium phosphate. The calcium phosphate removes prothrombin from the fibrinogen rich solution. This minimizes the likelihood of prothrombin conversion to thrombin which, if such reaction were to occur, could lead to the conversion of fibrinogen to fibrin. The calcium phosphate, in turn, is removed from the process by centrifugation and/or filtration. Additional techniques for removal of prothrombin are described by Murano (*Prothrombin and Other Vitamin K Proteins*, Vol. II, Seegers and Walz, eds., CRC Press, Inc. Boca Raton, Fl., 1986); Heystek, *et al.* (*Vox Sang.*, 25:113,

1973); Banowcliffe, *et al.* (*Vox Sang.*, 25:426, 1973); Chandra, *et al.* (*Vox Sang.*, 41:257, 1981); and Chanas, *et al.* (US. 4,465,623), incorporated herein by reference.

In a preferred embodiment, the dissolved cold-precipitate is warmed to about 23-27°C and contacted with lysine-Sepharose 4B. The fibrinogen rich fraction, which essentially does not bind to the lysine-Sepharose 4B, is separated from the affinity absorbent. The dissolved prothrombin-free cold-precipitate is contacted with lysine bound to a solid matrix, such as Sepharose, to allow adsorption of residual plasminogen in the cold-precipitate. The removal of plasminogen is important since it is a profibrinolytic zymogen which can break down fibrinogen and fibris molecules. The material which is not bound to the lysine matrix is then concentrated, preferably by the addition of polyethylene glycol (PEG). Polyethylene glycol (3000-8000 MW) is then added to a final concentration from about 3% to about 7%, preferably at about 4% (W/W). The PEG precipitate is dissolved and the pH adjusted to 8.6 ± 0.1 before clarification by filtration. When PEG is used for the concentration step, the weight-range of the PEG should be that which is non-toxic in humans. In an alternate embodiment of the invention, it is also possible to perform the PEG precipitation step prior to adsorption with the lysine matrix.

After filtration, the composition is treated with a virally inactivating effective amount of an agent, such as a detergent, which, typically, acts by disrupting the lipid envelope of such viruses as Hepatitis B, HIV, and HTLV. The term "virally inactivating effective amount" means that the concentration of detergent added to the composition is sufficient to inactivate essentially all virions which might be present. Of course, the concentration of detergent should not significantly inhibit the ability of the composition to function adequately in formation of the fibrin glue. Detergents which are useful can be selected from such recognized groups as anionic, cationic, and non-ionic detergents. Examples include sulfated alcohols and sodium acid salts, such

as sulfated oxyethylated alkylphenol (Triton W-30 and Triton X-100), sodium dodecylbenzensulfonate (Nacconol NR), sodium 2-sulfoethyl oleate (Igepon A), sodium cholate, sodium deoxycholate, sodium dodecylsulfonate, dodecyldimethylbenzylammonium chloride (Triton K-60), oxyethylated amines (Ethomeen), N-dodecylaminoethanesulfonic acid, ethylene oxide-propylene oxide condensates (Pluronic copolymers), polyoxyethylated derivatives of esters (Tween 80 and Polysorbate 80), polyoxyethylene fatty alcohol ethers (Brij 35), nonidet P-40 and Lubrox PX. In the preferred embodiment, where the protein concentration is 0.6%, Triton X-100 (final conc. 1.0%), and Polysorbate-80 (final conc. 0.3%) are combined with an organic solvent such as tri (N-butyl) phosphate (TNBP) (final conc. 0.3%) to inactivate viruses. In addition, chaotropic agent may also be utilized to inactivate viruses, providing the use of these agents does not result in significant loss of fibrinogen activity.

The concentration of organic solvent and detergent used in the practice of the preferred embodiments of the invention can vary, depending upon the composition to be treated, and upon the solvent or detergent selected. The alkyl phosphates are used in concentrations from about 0.10 mg/ml of mixture treated to 1.0 mg/ml, preferably between about 0.1 mg/ml to about 10 mg/ml. The amount of detergent or wetting agent utilized is not crucial since its function is to improve the contact between the organic solvent and the virus. For most of the nonionic materials which are useful, the wetting agent can vary from about 0.001% to 10%, preferably from about 0.01% to about 2% of the aqueous mixture, depending upon the amount of fatty material in the treated aqueous mixture. The amounts of solvent and detergent will also vary depending upon each other.

The prior art describes the addition of organic solvents and detergents to concentrated protein solutions in order to disrupt and inactivate lipid-enveloped viruses while preserving the structure and activity of the protein for example, U.S. Patent 4,540,573, which is incorporated herein by

reference. Organic solvents containing either Tween-80 or Triton X-100 have been shown to be effective reagents for killing viruses found in concentrated solutions of proteins without adversely affecting the activity of the proteins. However, the use of such solvent-detergent mixtures has traditionally been avoided because the subsequent removal of the detergent mixture from the concentrated protein has been shown to be difficult. As a result, other detergents, such as sodium cholate or sodium deoxycholate, have been more commonly used. These detergents can be removed from the composition by gel exclusion chromatography, for example, using Sephadex G-25. In any event, in selecting a given detergent, or mixture of detergents, it is important to avoid using those detergents which would denature fibrinogen in such manner as to prevent its ability to participate in clot formation.

In the preferred embodiment, DEAE diethylaminoethyl cellulose (DE 52) is the matrix utilized for the removal of the solvent/detergent from the fibrinogen composition. The fibrinogen binds to the DE 52 cellulose and, after thorough washing to remove unbound material and detergent, is eluted with 0.3M NaCl. Other ion exchange materials which can be utilized for removal of the solvent/detergent include virtually any of the commercially available anion exchange matrices, including celluloses and agaroses, such as polysulfated agaroses, specifically included but not limited to QAE (quaternary amine) derivatives, ecteola (epichlorohydrintriethanolamine), TEAE (triethylaminoethyl) derivatives, and AE (aminoethyl) matrices. The specific parameters for binding and eluting from these various ion exchange materials are known to those of skill in the art, or can be readily ascertained, without undue experimentation.

Upon elution from the ion exchange resin, the fibrinogen composition formulation is developed by the addition of such excipients as human serum albumin (HSA) and Polysorbate-80 added to enhance solubility. The addition of HSA, hydroxyethyl starch, dextran, or combinations thereof

enhances the stability of the final product. Preferably, the HSA and Polysorbate-80 are added at a concentration of 80mg and 15mg, respectively, per gram of protein in the eluate.

In a preferred embodiment, the fibrinogen composition of the invention is formulated prior to final concentration through ultrafiltration, lyophilization, or other conventional method, in order to enhance the solubility of the final product when reconstituted prior to therapeutic use. Typically, after formulation the bulk is concentrated from about 20% to about 50% of its original eluate volume, then diluted to the pre-concentration eluate volume. The bulk is then concentrated to a final protein concentration of 4±1g%(w/v) before sterile processing and lyophilization.

In the preferred embodiment, concentration is accomplished by ultrafiltration using a membrane with a molecular exclusion large enough to allow NaCl to be removed, but small enough to retain protein molecules. This filtration is most preferably performed using a membrane with a 30,000 MW exclusion. When the TFC composition of the invention is lyophilized, the pre-lyophilization volume is usually greater than the volume to which the lyophilizate is resuspended at time of use. Thus, when the composition is prepared as preferably described above involving ultrafiltration, the reconstituted volume following lyophilization is about 33% of the pre-lyophilization volume. In any event, the NaCl concentration in the reconstituted lyophilizate should preferably be about 0.1M to about 0.2M.

If desired, the compositions of the invention can be modified to include non-proteinaceous as well as proteinaceous drugs. The term "non-proteinaceous drugs" encompasses compounds which are classically referred to as drugs, such as mitomycin C, daunorubicin, and vinblastine as well as antibiotics.

The proteinaceous drugs which can be added to the fibrinogen compositions of the invention include immunomodulators and other

biological response modifiers. The term "biological response modifiers" is meant to encompass substances which are involved in modifying a biological response, such as the immune response or tissue growth and repair, in a manner which enhances a particular desired therapeutic effect, for example, the cytolysis of bacterial cells or the growth of epidermal cells. Examples of response modifiers include such compounds as lymphokines. Examples of lymphokines include tumor necrosis factor, the interleukins, lymphotoxin, macrophage activating factors, migration inhibition factor, colony stimulating factors, and the interferons. In addition, peptide or polysaccharide fragments derived from these proteinaceous drugs, or independently produced, can also be incorporated into the fibrinogen compositions of the invention. Those of skill in the art will know, or can readily ascertain, other substances which can act as proteinaceous or non-proteinaceous drugs.

The compositions of the invention can also be modified to incorporate a diagnostic agent, such as a radiopaque agent. The presence of such agents allow the physician to monitor the progression of wound healing occurring internally, such as at the liver, gall bladder, urinary tract, bronchi, lungs, heart, blood vessels, and spinal canal. Such compounds include barium sulfate as well as various organic compounds containing iodine. Examples of these latter compounds include iocetamic acid, iodipamide, iodoxamate meglumine, iopanoic acid, as well as diatrizoate derivatives, such as diatrizoate sodium. Other contrast agents which can be utilized in the compositions of the invention can be readily ascertained by those of skill in the art.

The concentration of drug or diagnostic agent in the composition will vary with the nature of the compound, its physiological role, and desired therapeutic or diagnostic effect. The term "therapeutically effective amount" means that the therapeutic agent is present in a sufficient concentration to minimize toxicity, but display the desired effect. Thus, for example, the

concentration of an antibiotic used in providing a cytolytic therapeutic effect will likely be different from the concentration of an immune response modulator where the therapeutic effect is to stimulate the proliferation of immune cells at the site of application of the fibrinogen complex. The term "diagnostically effective amount" denotes that concentration of diagnostic agent which is effective in allowing the fibrin glue to be monitored, while minimizing potential toxicity. In any event, the desired concentration in a particular instance for a particular compound is readily ascertainable by one of skill in the art.

The above disclosure generally describes the present invention. A further understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

PREPARATION OF TOPICAL FIBRINOGEN COMPLEX

Topical fibrinogen complex (TFC) was produced by the initial preparation of a cryoprecipitate of plasma. The cryoprecipitate for such use was prepared by two different techniques depending upon the physical form that the plasma was received.

In one technique, sealed plastic bottles of frozen plasma were thawed in a controlled environment by contact with a heat exchange medium, such as air or water. The thaw was controlled by programming the temperature and flow of the heat-exchange medium so that the maximum temperature of the plasma did not exceed +6°C. The containers were then opened and the contents pooled into a jacketed stainless steel thawing tank. In the thawing tank, the plasma is gently warmed (while being mixed) to melt the remaining ice. The thawed plasma was then pumped directly to a centrifuge or into a jacketed stainless steel holding tank where it was maintained at 2.5°C ± 3.5°C. The plasma was centrifuged to remove the cryoprecipitate. The cryoprecipitate, so prepared, may be stored at or below -25°C or immediately processed to Antihemophilic Factor (Human). The cryo-poor plasma was collected in a jacketed stainless steel reaction tank.

Alternatively, cryoprecipitate was prepared by placing sealed plastic bags of frozen plasma in a liquid nitrogen bath for several seconds. The bags were removed from the bath and the crisp, cracked bags were stripped from the plasma. The plasma was then placed into a jacketed stainless steel thawing tank. Alteratively, sealed plastic bags of frozen plasma were arranged so as to warm the bags so that the frozen plasma would break away from the plastic. The containers were then opened and the contents pooled into a jacketed stainless steel thawing tank. In the thawing tank the plasma was gently warmed, while being mixed, to melt the remaining ice.

The thawed plasma was pumped directly to a centrifuge or into a stainless steel holding tank where it was maintained at 2.5°C ± 3.5°C. The plasma was centrifuged to remove the cryoprecipitate. The cryoprecipitate, so prepared, may be stored at or below -25°C, or immediately be processed to Antihemophilic Factor (Human). The cryo-poor plasma is collected in a jacketed stainless steel reaction tank.

After the cryoprecipitate was prepared, it was dissolved in distilled water at 20°C to 35°C. This part of the protocol is illustrated schematically in FIGURE 1. Sufficient calcium chloride was added to obtain a minimum calcium concentration of about 40 μM and the pH adjusted to 6.8 ± 0.3. This solution was cooled at 10°C ± 2°C while mixing. The precipitate which forms was removed by centrifugation (5600g-7200g). The precipitate may be stored at or below -60°C or processed directly to Topical Fibrinogen Complex (Human). The precipitate is then suspended in Process Solution I at a ratio of four liters of Process Solution I per kg of precipitate. Process Solution I comprises: 0.5M glycine, 0.5M sodium chloride, and 0.1M sodium citrate; pH adjusted to 7.2 ± 0.1 with NaOH and, Protease Inhibitor: 0.75-1.75 μM PPACK (D-phe-L-pro-L-arg-chloromethyl ketone) or equivalent; 0.6 ± 0.1 U/ml Heparin. The temperature was adjusted to 24-32°C and the suspension stirred for approximately one hour.

After the precipitate was suspended in Process Solution I, the suspension was transferred into a tank containing 200 l of process Solution II at 10-15°C and stirred for at least 30 minutes. Process Solution II comprises: 7 ± 1 mM sodium phosphate monobasic monohydrate. 18 ± 2 mM sodium phosphate dibasic heptahydrate, calcium phosphate tribasic 0.25% (w/v). The suspension was then allowed to settle undisturbed for at least 30 minutes. The suspension may be centrifuged (5600g-7200g) at this step to remove some precipitate. The suspension was then clarified by filtration first through a 0.45μ filter, then through a 0.2μ filter.

The filtrate was warmed to 23-27°C and applied to a Lysine Sepharose 4B column or equivalent. The gel was packed in a chromatography column, prepared with 10 column volumes of 0.1M acetic acid, 3 column volumes of distilled water, and equilibrated with 5 column volumes of Process Solution III (25 mM phosphate buffer: 7 ± 1 mM sodium phosphate monobasic monohydrate, 18 ± 2 mM sodium phosphate dibasic heptahydrate), respectively. If desired, the column can be reused upon regeneration with 3 column volumes of 1.0M NaCl, 3 column volumes of distilled water, 3 column volumes of 0.1 N acetic acid, and 3 column volumes of distilled water, respectively. For long term storage, the column was washed with 3 column volumes of 20% ethanol in distilled water and stored. The unbound material was collected in a tank. The column was washed with at least two gel volumes of Process Solution III. The unbound fractions were pooled. Next, the temperature of the bulk containing the pooled unbound fractions was adjusted to 14 ± 4°C. Polyethylene glycol 3350 was added to a final concentration of 4% (w/w). The suspension was mixed for at least 30 minutes and the precipitate removed by centrifugation (8700g) at 10-18°C. The resulting PEG precipitate was dissolved in Process Solution IV (39 mM tris-phosphate with pH adjusted to 8.6 ± 0.1 with phosphoric acid) approximately 15 l/kg precipitate. The protein concentration of the suspension was adjusted to 0.6 ± 0.2 g% (w/v), then the suspension was clarified by filtration (0.2μ). A mixture of Triton X-100, tri (N-butyl) phosphate (TNBP) and Polysorbate-80 was added to the solution to a final concentration of 1.0% (v/v), 0.3% (v/v), and 0.3% (v/v), respectively. The protein-detergent solution was mixed for 1 hour.

The bulk solution was then applied to a chromatography column containing DE 52 ion exchange cellulose resin or equivalent. The resin was packed in a chromatography column and regenerated with 3 column volumes of 1.0M NaCl, 3 column volumes of 0.5M HCl, 3 column volumes of 0.9% saline, 3 column volumes of 0.5M NaOH, respectively, and equilibrated with 3 column volumes of 0.5M tris-phosphate buffer (pH adjusted to 8.6), and 3 column

within the delivery device and longer times lead to a loose mixture of the components that flows with ill-defined direction.

In performing these experiments, a clean pyrex glass plate was positioned at ~30° from the horizontal axis. A 2" line was drawn on the underside to define the area of FS application.

Human Fibrinogen was tested at 50-130 mg/ml total protein with Bovine Thrombin (Armour Pharmaceutical Co.) at 100-1000 NIH U/ml without Ca^{++}. Fibrinogen concentration was considered to equal the value of total proteins. The effect of [Ca^{++}] was studied at 10, 20, 40, and 60 mM.

The Fibrin Sealant was delivered using an experimental dual syringe device (Fenwal) following the 2" line described above, starting at the upper end and moving downward.

Table 1 summarizes the data obtained by testing Fibrinogen at 50-130 mg/ml with Thrombin at 100-1000 NIH U/ml in the absence of Calcium. Each data point is the average of four determinations.

TABLE 1

AVERAGE CLOTTING TIMES IN SECONDS (±SD)

	Thrombin in NIH U/ml			
Total Protein (mg/ml)	100	250	500	1000
50	5.3±1.3	2.5±0.4	2.3±0.4	1.2(N=1)
90	3.8±0.4	2.4±0.4	1.9±0.4	1.4(N=1)
110	5.3±1.6	2.2±0.5	1.6±0.1	*
130	3.6±0.6	1.8±0.2	0.8(N=1)	*

* Not Determined - clotting occurred too fast for time measurement.

As Table 1 shows, clotting times at the lower thrombin concentration (e.g., 100 NIH U/ml) and low protein content (e.g., 50 mg/ml), were long. The mixture was also observed to be "runny". Higher concentrations of thrombin (e.g., 1000 NIH U/ml) generally clotted within the delivery device and therefore were considered unsuitable. Addition of $CaCl_2$ improved the appearance of the clot and generally shortened the clotting time.

Table 2 shows the effect of $[CA^{++}]$ in the range of 0-60 mM. $CaCl_2$ solution was used to reconstitute the thrombin so the final $[Ca^{++}]$, in the 1:1 mixture of FS is half that reported in the Table.

TABLE 2

EFFECT OF [Ca^{++}] ON TIME TO CLOT

Total Protein mg/ml	Thrombin in NIH U/ml 100	250	500
1. [Protein]=50 mg/ml			
0 mM	4.3±0.7	2.6±0.2	2.0±0.6
10 mM	2.4±0.3	1.8±0.2	*
20 mM	2.3±0.2	1.2±0.5	*
40 mM	2.6±0.2	*	*
60 mM	3.0±0.5	*	*
2. [Protein]=90 mg/ml			
0 mM	4.1±1.1	2.2±0.2	1.8±0.3
10 mM	2.9±0.3	2.8±0.3	*
20 mM	2.0±0.4	2.6±0.1	*
40 mM	2.5±0.2	1.9±0.1	*
60 mM	3.2±0.1	*	*
3. [Protein]=110 mg/ml			
0 mM	4.8±1.8	2.4±0.5	1.7±0.3
10 mM	2.9±0.4	2.4±1.2	*
20 mM	2.6±0.2	*	*
40 mM	2.6±0.7	*	*
4. [Protein]=130 mg/ml			
0 mM	3.1±0.8	1.5±0.1	*

* Not Determined - clotting occurred too fast for time measurement

Based on the above results, it was concluded that a protein range of 90-130 mg/ml and a thrombin concentration of 250-500 NIH U/ml are appropriate for further studies. It was also apparent that additional calcium ion was needed to enhance the clot. Consequently, Ca^{++} concentration was included as a variable in later evaluations.

B. Rate of Cross-Linking

These studies focused on determining the role of Ca ions and the effect of $[Ca^{++}]$ on the extent of fibrin polymerization as well as the rate of cross linking with time due to fibrin polymerization.

The cross-linking reaction of fibrin was tested in a system under reduced conditions which utilized SDS/PAGE. The resolving gels at 7.5% and the stacking gels at 3.75% were cast as described by Schwartz, *et al.* (*Journal of Clinical Investigation*, 50:1506, 1971).

Thrombin (Armour Pharmaceutical) was reconstituted with or without $CaCl_2$ solution at the desired molarity, i.e., 0, 2, 6, 12, 20, 40, or 60 mM. Fibrinogen was reconstituted with water, quickly mixed with the thrombin in a 12 x 75 mm test tube and sampled at the appropriate time periods for the studies. The clots were rinsed with 0.15M NaCl then dissolved in three times the clot volume of 9M urea containing 3% SDS and 3% ß-mercaptoethanol by boiling in a water bath at 95 ± 5°C. The dissolved clot solutions were then stored at 5°C until the gel electrophoresis was performed.

The effect of $CaCl_2$ concentration was tested with thrombin at 500 NIH U/ml and fibrinogen at 90 mg/ml and 130 mg/ml after 10 minutes clotting time. The effect of Ca^{++} concentration on the rate of disappearance of the γ-band to form the γ-γ dimer is shown in FIGURES 2 and 3.

As illustrated in FIGURES 2 and 3, the presence of Ca^{++} is necessary for complete fibrin polymerization. As calcium ion in the range of 20-60 mM give comparable results, a midpoint concentration of 40 mM was chosen to ensure optimal polymerization. Also, there was no significant difference in fibrin polymerization between the two fibrinogen concentrations (90 mg/ml and 130 mg/ml) when measured at the time point studied (10 minutes).

Four combinations of thrombin at 250 NIH U/ml or 500 NIH U/ml in 40 mM $CaCl_2$ and fibrinogen at 90 mg/ml or 130 mg/ml were tested to study the effect of varying the concentrations of the components. Samples were taken for gel electrophoresis after 10 minutes of clotting time. Gel electrophoresis of the four combinations of thrombin (in 40 mM $CaCl_2$) and fibrinogen did not reflect any significant differences at the time point studied (10 minutes).

The time study was conducted and sampled over 24 hours. FIGURE 4 shows the fibrin polymerization reaction as it progresses through a 24 hour period. Formation of the γ-γ dimer occurs very rapidly in the presence of Ca^{++} (within one minute) as is shown in FIGURE 4. The α polymer is not detectable by this system until 10 minutes incubation time. As the α polymer increases with increasing incubation time (up to 24 hours) the α monomer band shows a corresponding decrease in intensity. To summarize, the time study demonstrates that initial polymerization (γ-γ dimer) occurs almost instantaneously as the reactants are mixed, with the α polymers forming more slowly. From this study it can be concluded that the presence of Ca ion is necessary for polymerization and that the results are similar to those previously reported in the literature (T. Seelich, *J. Head & Neck Pathol.*, 3:65-69, 1982; M. Schwartz, *et al.*, *J.Clinical Inv.*, 50:1506-1513, 1971).

C. Tensile Strength

The tensile strength of the fibrin sealant was evaluated by applying strain to the clot until rupture of the bulk material was observed and measuring the force needed in a tensile stress-strain system. In addition, the change in rupture stress as a function of varying the components in the polymerization mixture used to produce the sealant was studied.

To study the tensile strength of the Fibrin Sealant, a mold was designed based on that described by Nowotny, *et al.* (*Biomaterials*, 2:55, 1981) with some modifications. The newly designed mold was fabricated of transparent plastic to facilitate visual inspection of clot formation. Clotting was allowed to proceed in disposable clot holders for ease of cleaning.

The clot holders were obtained by cutting plastic disposable transfer pipets (SAMCO, San Fernando Mfg. Co.). Two small pieces of moistened sponge were used to anchor the clotting mixture at both ends. Disposable clot holders with the sponges in place were inserted through the end holders and into the mold, (end holders were included in the mold). A tensometer instrument (T10, Monsanto) was used to measure and record the peak rupture stress of the clots. Adapters for the T10 grippers were fabricated to hold the end holders.

The clots were formed by injecting equal volumes of fibrinogen and thrombin (with or without $CaCl_2$) using a dual syringe administration device (Fenwal) and a 3 inch 22 gauge needle. All bubbles were removed prior to placing the syringes in their holder. The needle was inserted through one sponge "top", through the mold and into the other sponge "bottom". Parafilm (American Can Co.) placed under the entire mold prevented leakage of excess mixture. As the clotting mixture filled the mold, the needle was withdrawn.

Approximately 2 to 5 minutes before testing, the clot was removed from the mold and placed in the T10 grippers. At testing time, the clot was stretched at a rate of 100 mm/min. The gauge length was set (somewhat arbitrarily) at 6.0 cm and the cross sectional area of the clot was 0.049 cm^2. The T10 reported the stress values in Kgf/cm^2.

TABLE 3

EFFECT OF $CaCl_2$ ON TENSILE STRENGTH OF FIBRIN SEALANT

Fgn.* Conc. mg/ml	$CaCl_2$ mMol	Tensile Strength KgF/cm^2 @ 250 NIH U/ml Thrombin (N)	Tensile Strength KgF/cm^2 @ 500 NIH U/ml Thrombin (N)
90	0	0.93±0.136 (9)	1.23±0.165 (8)
110	0	1.07±0.150 (10)	1.29±0.237 (9)
130	0	0.94±0.157 (14)	1.36±0.177 (16)
90	10	1.65±0.380 (12)	1.92±0.350 (8)
110	10	2.60±0.540 (8)	2.80±0.610 (8)
130	10	2.35±0.760 (11)	3.24±1.170 (8)
90	20	1.65±0.406 (8)	2.15±0.540 (8)
110	20	2.26±0.530 (8)	4.11±1.080 (8)
130	20	2.23±0.489 (8)	3.54±1.030 (8)
90	40	1.74±0.410 (8)	2.36±0.390 (11)
110	40	2.36±0.660 (8)	3.79±0.626 (8)
130	40	2.63±0.670 (12)	4.05±0.940 (9)
90	60	2.10±0.38 (8)	2.29±0.450 (8)
110	60	3.00±0.690 (8)	4.13±0.896 (8)
130	60	3.69±0.787 (10)	3.57±1.160 (9)

* Fibrinogen Lot #2830R129

Measurements of tensile strength (peak stress) were taken 10 minutes after injection of the clotting mixture. The results in Table 3 show the effect of varying the $CaCl_2$ concentration when thrombin is 250 NIH U/ml or 500 NIH U/ml. Three concentrations of fibrinogen (90, 110, and 130 mg/ml) were tested. Each measurement of peak stress was the average of N determinations. A minimum of 8 readings were taken per point.

As shown in Table 3, in the absence of Ca^{++}, the clots had the lowest peak stress values at both thrombin concentrations and at all fibrinogen levels. Addition of Ca ions at 10-60 mM increased the tensile strength for all thrombin/fibrinogen concentrations. Generally, higher values were observed at the higher thrombin concentration, i.e., 500 NIH U/ml, which also gave somewhat similar values for $[Ca^{++}]$ in the range of 20-60 mM. The lowest standard deviation (SD) values were observed at 40 mM $CaCl_2$.

A second lot of human fibrinogen was tested to confirm these findings and the data were compared at 40 and 60 mM $CaCl_2$ and 500 NIH U/ml thrombin. The results of testing a second lot of fibrinogen showed generally similar values of peak stress particularly at the higher fibrinogen concentrations of 110 and 130 mg/ml and also showed higher values at 90 mg/ml.

Time studies of the clot tensile strength were performed over a 24 hour time period using fibrinogen at 90 mg/ml and 130 mg/ml with thrombin concentration at 500 NIH U/ml and $CaCl_2$ at 40 mM. Over a 24 hour period, the tensile strength showed no significant decrease in value. A gradual increase in peak stress was expected to occur as cross linking continued with time.

D. Clot Lysis Studies

The length of time that a fibrin clot will remain solid when incubated at 37°C under sterile, moist conditions, with and without a plasminogen activator, was determined. Also tested was the effect on the clot longevity of adding protease inhibitor (Aprotinin) to the reaction mixture.

Sterile human fibrinogen solution was prepared using one of the following diluents:

a. sterile water (i.e., zero KIU/ml Aprotinin)
b. Aprotinin solution at 1000 KIU/ml
c. Aprotinin solution at 3000 KIU/ml

to yield one of three concentrations; 90, 110, or 130 mg/ml of total protein.

Thrombin was prepared by reconstituting with a 40 mM $CaCl_2$ solution to produce either a 250 or 500 NIH U/ml. Thus, six combinations of thrombin and fibrinogen were tested. Urokinase (Abbott) was prepared at 5 U/ml in normal saline.

Fibrin clots were formed by mixing equal volumes of fibrinogen (in H_2O or Aprotinin) and thrombin (in $CaCl_2$ solution) in cylindrical silicone tubing (5 mm inner diameter). The mixture was delivered using a dual syringe administration device (Fenwal). All the delivery devices and the silicone tubing were sterilized by autoclaving.

A 10 cm length of silicone tubing was sealed at one end using parafilm. Holding the tubing about 10° from vertical, the Fenwal device was used to inject the fibrinogen and thrombin rapidly into the tubing with the (22 g) needle tip barely penetrating the parafilm. After the clot had solidified for 20 minutes, the 10 cm silicone tubing was cut into 3 cm lengths to yield a clot volume of 590 μl. Each 3 cm segment was cut in half and the two halves were placed in one well of a sterile 24-well plate (Corning). Any segment that was found to contain air bubbles was discarded. The clot was extruded from the tubing by gently squeezing the tube at one end. It was rinsed with 1 ml of sterile saline then 1 ml of either urokinase or saline was added to the well and the plate was placed in a sterile, moist, 37°C incubator. Every 24 hours, the supernatants from each well were removed for testing using the Fibrin(ogen) Degradation Products agglutination kit (Baxter Dade).

The urokinase and saline solutions were replaced daily with fresh reagents before returning the plate to the 37°C incubator. All preparations and sampling of supernatants were performed under sterile conditions. The clots were visually inspected and their appearance noted. After 14 days the experiment was terminated. Total number of conditions tested was 36 and all conditions were performed in duplicate.

TABLE 4

CLOT LYSIS TIMES
[+ UROKINASE, NO APROTININ]

CONDITION	TIME CLOT LOST CYLINDRICAL SHAPE
130 mg/ml fibrinogen + 500 U/ml thrombin	Day 10
130 mg/ml fibrinogen + 250 U/ml thrombin	Day 8
110 mg/ml fibrinogen + 500 U/ml thrombin	Day 7
110 mg/ml fibrinogen + 250 U/ml thrombin	Day 11
90 mg/ml fibrinogen + 500 U/ml thrombin	Day 8
90 mg/ml fibrinogen + 250 U/ml thrombin	Day 8

Table 4 summarizes the clot lysis time (defined as the time clots lost their cylindrical shape) in the presence of urokinase when no Aprotinin was included. The observed range was 7-11 days with a mean of 8.66 ± 1.5d. Measurements of Fibrin(ogen) Degradation Products (FDP) showed a peak in activity that generally corresponded to or soon followed the time when the clots lost their well-defined shape.

When urokinase was deleted and the clots were incubated in normal saline only, in presence or absence of Aprotinin, the clots maintained their integrity during the entire observation period (i.e., 14 days). FDP measurements confirmed the absence of significant clot lysis.

This study shows that when the clot is not influenced by any plasminogen activators *in situ*, it should be expected to last for at least 14 days. When urokinase is present, the clots last a minimum of 7 days. These time periods may be sufficient for the healing mechanism to play its natural role. Thus, based on these results, it can be concluded that the trace levels of plasminogen in the fibrinogen preparations do not adversely affect the clot longevity and that the addition of a protease inhibitor, such as Aprotinin, is not necessary.

EXAMPLE 3

TFC *in vivo* TESTING

Studies were done to evaluate the optimal concentration of TFC using an *in vivo* model. Swiss Webster mice (20-25 g) were arranged in 10 groups of five for testing. In the protocol which was utilized, each animal was anesthetized, weighed, and a small piece of skin was removed from the back of the animal. The skin specimen was dipped in a saline solution and attached to a Gottlob device. Equal volumes of TFC and thrombin at various concentrations (Table 2) were then added simultaneously to the wound, the skin replaced onto the animal, and held in place for approximately two minutes.

The anesthetized animal was placed face down on a platform which was then positioned on a tensometer (Monsanto Company) and the Gottlob device attached to the grippers. The tensometer parameters were set to: (1) area: 1.76 cm^2; (2) speed: 10.0 mm/min.; (3) gauge: 1.0 cm; (4) stress

range: 500.0%. The force required to separate the piston (with the skin specimen) from the back of the animal was recorded in g/cm^2. The data from these experiments were statistically evaluated using RS1/Discover software (BBN Software Corp., Cambridge, MA).

TABLE 5

GROUP	N	TFC	THROMBIN
1	5	130	500
2	5	130	375
3	5	130	250
4	5	110	500
5,6	10	110	375
7	5	110	250
8	5	90	500
9	5	90	375
10	5	90	250

Analysis of the results of this study indicated that TFC at 120-130 mg/mL and thrombin at 250 U/mL gave maximal adhesion responses.

The ability of the clot to adhere to tissue *in vivo* is important in maintaining hemostasis. In this experiment, a maximum adhesion response occurred within the range of reagents tested, confirming the *in vitro* findings of Example 2.

The preceding examples and description are provided to assist in understanding the present invention and, as such, are intended to be exemplary only, not limiting. Those of skill in the art will recognize that other

materials or methods may be used, depending on the circumstances, and still remain within the spirit and scope of the present invention.

CLAIMS

1. A method for preparing a hemostasis promoting composition derived from human plasma or plasma fractions wherein the human plasma or plasma fractions comprise fibrinogen, Factor VIII, and plasminogen, comprising:
   (a) providing a cryoprecipitated plasma preparation from the human plasma or plasma fractions;
   (b) separating the cryoprecipitate from the cryoprecipitated plasma preparation;
   (c) forming a cold-precipitate by dissolving the cryoprecipitate in a medium and cooling the medium, the cold-precipitate having significantly less Factor VIII than the cryoprecipitate;
   (d) suspending the cold-precipitate of step (c) the suspension then added to a medium comprising calcium phosphate;
   (e) treating the supernatant obtained from the suspension in step (d) by affinity-chromatography to allow plasminogen to adsorb thereon;
   (f) collecting the fraction essentially free of plasminogen;
   (g) contacting the fraction of step (f) with a virally inactivating effective amount of an antiviral agent;
   (h) removing the antiviral agent from the virally inactivated material obtained in step (g); and
   (i) recovering a fibrinogen-containing composition.

2. The method of claim 1, wherein the cryoprecipitate in step (c) is initially dissolved in water having a temperature from about 20°C to about 35°C and the cooling is maintained at a temperature of 10°C ± 5°C.

3. The method of claim 1, wherein calcium ion is added to the dissolved cryoprecipitate in step (c) at a concentration sufficient to enhance the precipitation of fibrinogen.

4. The method of claim 3, wherein the calcium ion is in the form of a calcium salt.

5. The method of claim 4, wherein the calcium salt is $CaCl_2$.

6. The method of claim 5, wherein the $CaCl_2$ is added to a final concentration from about 0.001 mM to about 1 mM.

7. The method of claim 6, wherein the $CaCl_2$ is added to a final concentration of about 0.040 mM.

8. The method of claim 1, further including the step of (c') prior to step (d), wherein the step (c') comprises treating the cold-precipitate with a protease inhibitor in a concentration sufficient to inhibit thrombin activity.

9. The method of claim 8, wherein the protease inhibitor is selected from the group consisting of PPACK, heparin cofactor II, hirudin, and anti-thrombin III (AT III).

10. The method of claim 9, wherein the concentration of PPACK is from about 0.75 μM to about 1.75μM.

11. The method of claim 1, wherein in step (e) the affinity-chromatography consists of a lysine-bound solid matrix as the adsorbant.

12. The method of claim 11, wherein the solid matrix is agarose.

13. The method of claim 1, further including the step of (f') prior to step (g), wherein the step (f') comprises treating the fractions by addition of polyethylene glycol (PEG).

14. The method of claim 13, wherein the PEG has a molecular weight from about 3,000 to about 8,000.

15. The method of claim 14, wherein the PEG has a molecular weight of about 3350.

16. The method of claim 13, wherein in step (f') the PEG is added to a final concentration from about 3% to about 7%.

17. The method of claim 13, wherein in step (f') the PEG is added to a final concentration of about 4% (w/w).

18. The method of claim 1, wherein in step (g) the anti-viral agent is a detergent.

19. The method of claim 18, wherein the detergent is non-ionic.

20. The method of claim 19, wherein the concentration of the detergent is from about 0.001% (v/v) to about 10% (v/v).

21. The method of claim 20, wherein the concentration of detergent is from about 0.01% (v/v) to about 2% (v/v).

22. The method of claim 18, wherein a mixture of detergents is utilized.

23. The method of claim 22, wherein the mixture is non-ionic.

24. The method of claim 23, wherein the mixture includes a sulfated oxyethylated alkylphenol and a polyoxyethylated ester derivative.

25. The method of claim 24, wherein the sulfated oxyethylated alkylphenol is Triton X-100.

26. The method of claim 24, wherein the polyoxyethylated ester derivative is Polysorbate-80.

27. The method of claim 23, wherein the total final concentration of detergent mixture is from about 1% (v/v) to about 2% (v/v).

28. The method of claim 23, wherein the total final concentration of detergent mixture is from about 1% (v/v) to about 1.5% (v/v).

29. The method of claim 23, wherein the total final concentration of detergent mixture is from about 1.2% (v/v) to about 1.4% (v/v).

30. The method of claim 25, wherein the final concentration of Triton x-100 is from about 0.8% (v/v) to about 1.2% (v/v).

31. The method of claim 26, wherein the final concentration of Polysorbate-80 is from about 0.2% (v/v) to about 0.4% (v/v).

32. The method of claim 1, wherein in step (g) the anti-viral agent is an organic solvent.

33. The method of claim 32, wherein the organic solvent is an alkyl phosphate.

34. The method of claim 33, wherein the alkyl posphate is tri (n-butyl) phosphate.

35. The method of claim 34, wherein the final concentration of tri (n-butyl) phosphate is from about 0.2% (v/v) to about 0.4% (v/v).

36. The method of claim 18 or claim 32, wherein the anti-viral agent is removed by adsorbing fibrinogen on an anionic exchange resin and by washing the anti-viral agent not bound to the resin.

37. The method of claim 36, wherein the anionic exchange moiety is diethylaminoethyl (DEAE).

38. The method of claim 37, wherein the DEAE is attached to cellulose.

39. The method of claim 38, wherein the anion exchange matrix is DE 52 cellulose.

40. The method of claim 36, wherein the material bound to the anion exchange resin is eluted with about 0.25 M to about 0.40 M NaCl.

41. The method of claim 1, which further comprises the steps of:
    (j) adding a stabilizer and/or a solubilizer to the recovered composition of step (i);
    (k) concentrating the composition of step (j) to about 20% to about 50% of its original volume;
    (l) diluting the concentrate of step (k) to its preconcentration volume;
    (m) concentrating the composition of step (l) to about 3 g% to about 5 g% (w/v), and
    (n) sterile processing the composition.

42. The method of claim 41, wherein in step (n) the composition is additionally lyophilized.

43. The method of claim 41, wherein the pre-lyophilization volume of the composition is about 3 times the volume of the composition after the lyophilizate is resuspended.

44. The method of claim 43, wherein the final concentration of NaCl after resuspension of the lyophilizate is about 0.1 M to about 0.2 M.

45. The method of claim 41, wherein in step (j) the stabilizer is human serum albumin (HSA).

46. The method of claim 45, wherein the HSA is from about 70mg to about 100mg per gram of protein in the eluate.

47. The method of claim 41, wherein in step (j) the solubilizer is a polyoxyethylated ester derivative.

48. The method of claim 47, wherein the polyoxyethylated ester derivative is Polysorbate-80.

49. The method of claim 48, wherein the Polysorbate-80 is from about 10mg to about 20mg per gram of protein in the eluate.

50. The method of claim 41, wherein in step (k) and in step (m), the concentration is accomplished by ultrafiltration.

51. The method of claim 50, wherein the ultrafiltration utilizes a membrane with a 30,000 molecular weight cut-off.

52. A hemostasis promoting composition derived from human plasma or plasma fractions characterized as:
    (a) having from about 75% to about 95% fibrinogen;
    (b) from about 10 to about 40 units/ml F XIII;
    (c) being virally inactive;
    (d) being substantially non-pyrogenic;
    (e) having a maximum of about 10 μg/ml plasminogen; and
    (f) containing essentially only protein encoded by a human DNA sequence.

53. The composition of claim 52, wherein the composition is lyophilized.

54. The composition of claim 52, which further comprises a stabilizer and/or a solubilizer.

55. The composition of claim 54, wherein the stabilizer is HSA.

56. The composition of claim 55, wherein the HSA is at a concentration from about 0.5 g% to about 1.5 g%.

57. The composition of claim 54, wherein the solubilizer is a polyoxyethylated ester derivative.

58. The composition of claim 57, wherein the polyoxyethylated ester derivative is Polysorbate-80.

59. The composition of claim 58, wherein the Polysorbate-80 is from about 0.1% to about 0.3%.

60. The composition of claim 52, which further comprises a therapeutically effective amount of a drug.

61. The composition of claim 60, wherein the drug is proteinaceous.

62. The composition of claim 61, wherein the proteinaceous drug is a colony stimulating factor or a growth factor.

63. The composition of claim 60, wherein the drug is non-proteinaceous.

64. The composition of claim 63, wherein the non-proteinaceous drug is an antibiotic.

65. The composition of claim 52, which further comprises a diagnostically effective amount of a diagnostic agent.

66. The composition of claim 65, wherein the diagnostic agent is a radiopaque agent.

67. The composition of claim 66, wherein the radiopaque agent is an iodinated organic compound.

68. The composition of claim 67, wherein the iodinated organic compound is a diatrizoate derivative.

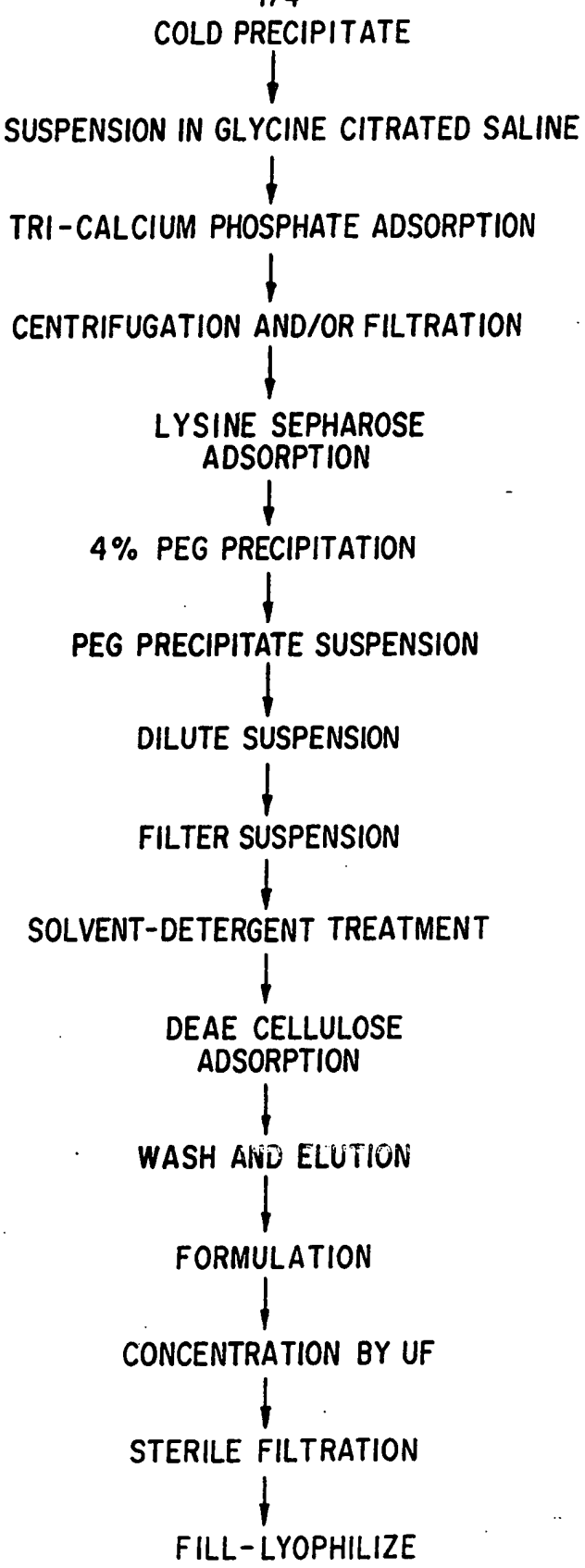
COLD PRECIPITATE
SUSPENSION IN GLYCINE CITRATED SALINE
TRI-CALCIUM PHOSPHATE ADSORPTION
CENTRIFUGATION AND/OR FILTRATION
LYSINE SEPHAROSE ADSORPTION
4% PEG PRECIPITATION
PEG PRECIPITATE SUSPENSION
DILUTE SUSPENSION
FILTER SUSPENSION
SOLVENT-DETERGENT TREATMENT
DEAE CELLULOSE ADSORPTION
WASH AND ELUTION
FORMULATION
CONCENTRATION BY UF
STERILE FILTRATION
FILL-LYOPHILIZE

FIG. 1

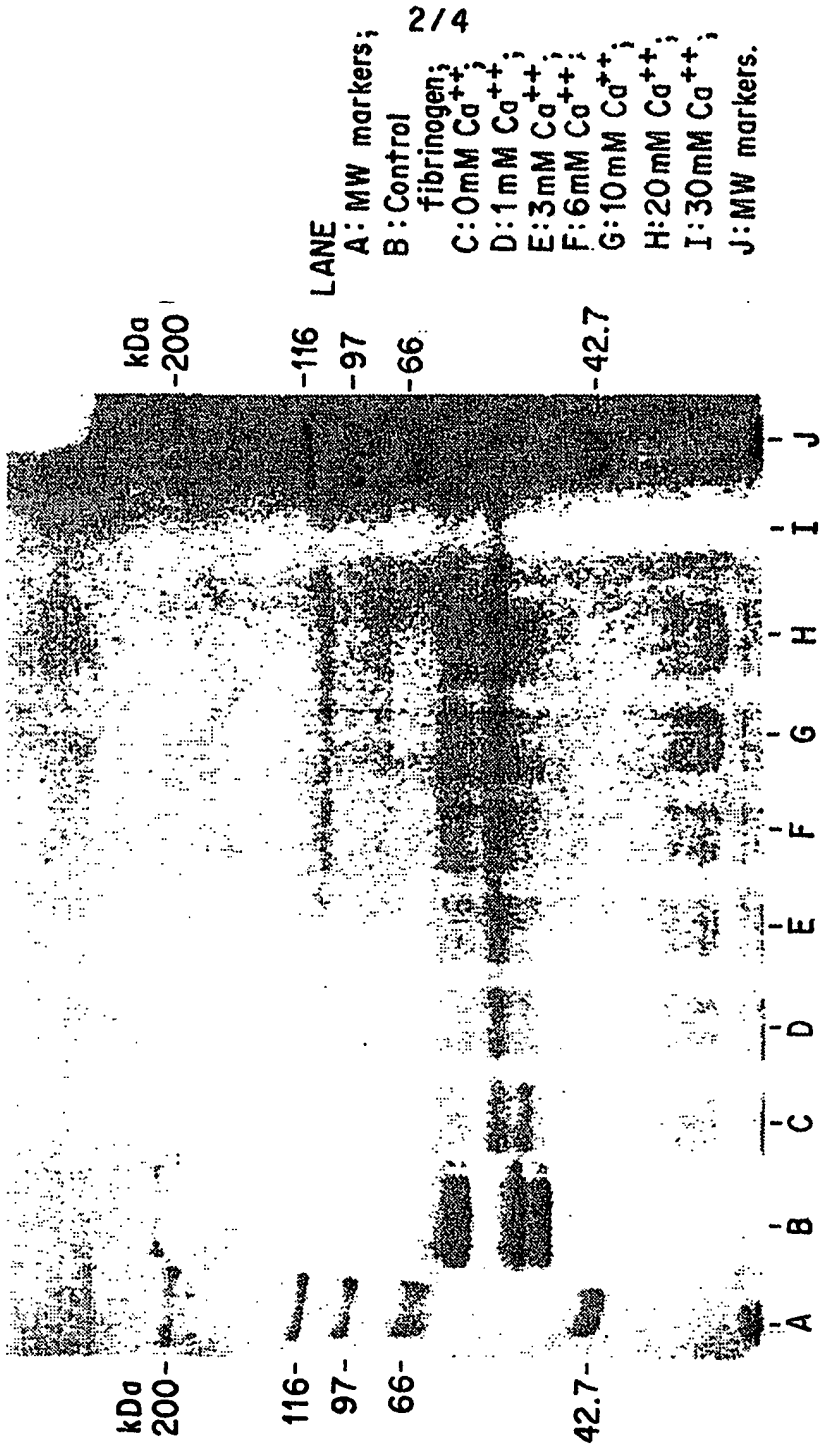
LANE
A: MW markers;
B: Control fibrinogen;
C: 0mM Ca++;
D: 1mM Ca++;
E: 3mM Ca++;
F: 6mM Ca++;
G: 10mM Ca++;
H: 20mM Ca++;
I: 30mM Ca++;
J: MW markers.
kDa
200
116
97
66
42.7
A
B
C
D
E
F
G
H
I
J

FIG. 2

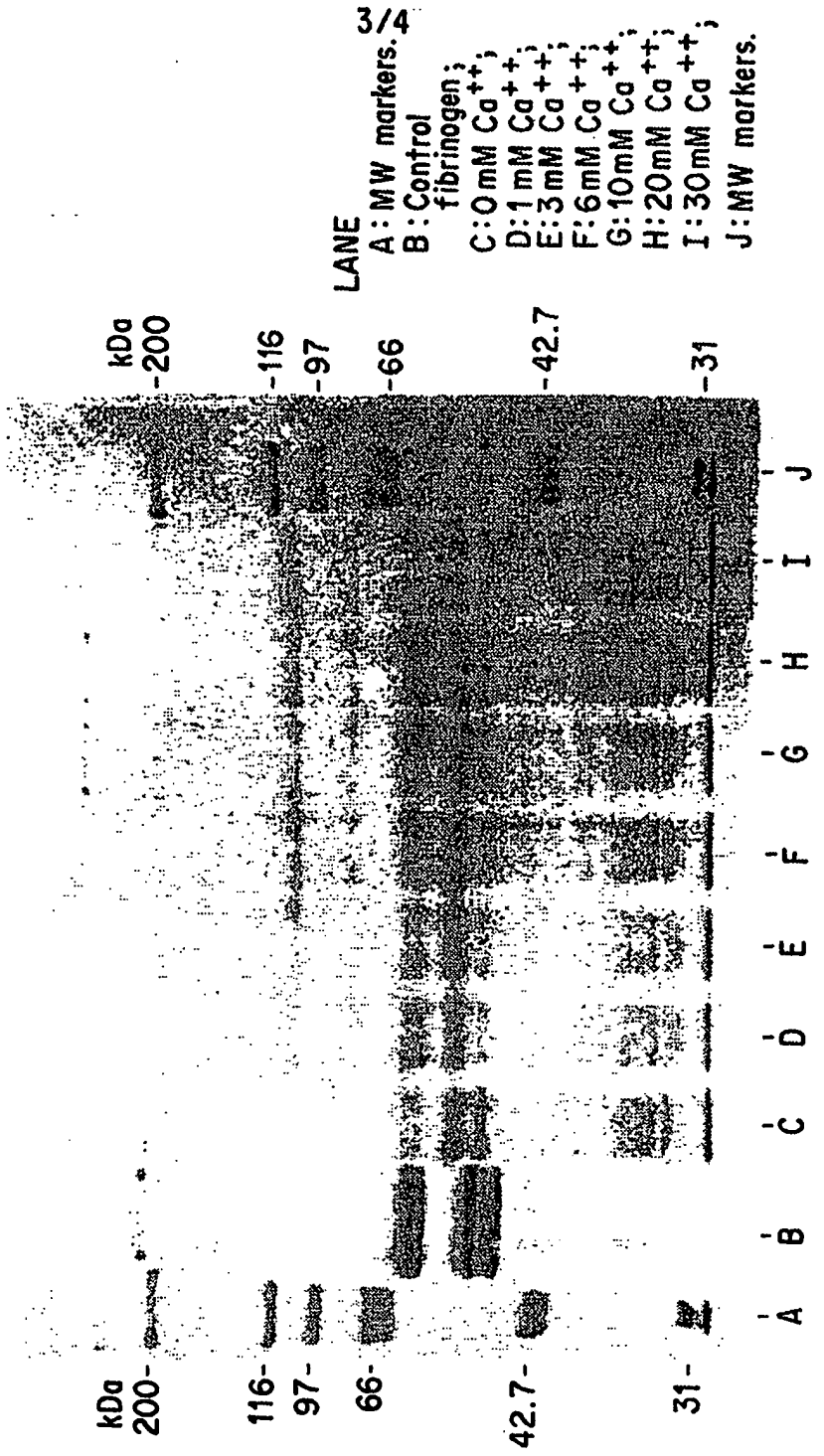
LANE
A: MW markers.
B: Control fibrinogen;
C: 0 mM Ca++;
D: 1 mM Ca++;
E: 3 mM Ca++;
F: 6 mM Ca++;
G: 10 mM Ca++;
H: 20 mM Ca++;
I: 30 mM Ca++;
J: MW markers.
kDa
200
116
97
66
42.7
31
A B C D E F G H I J

FIG. 3

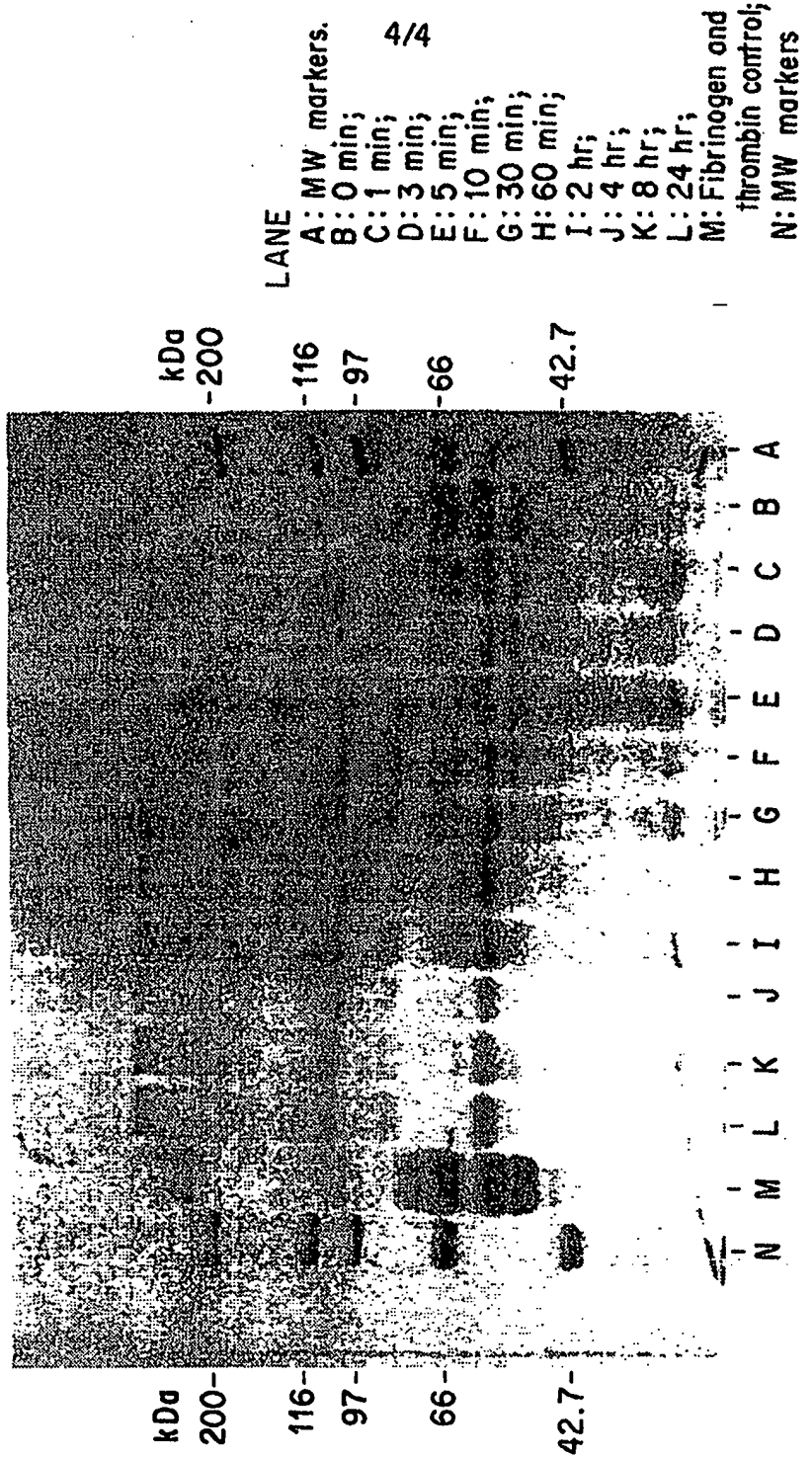
LANE
A: MW markers.
B: 0 min;
C: 1 min;
D: 3 min;
E: 5 min;
F: 10 min;
G: 30 min;
H: 60 min;
I: 2 hr;
J: 4 hr;
K: 8 hr;
L: 24 hr;
M: Fibrinogen and thrombin control;
N: MW markers
kDa
200
116
97
66
42.7
N M L K J I H G F E D C B A

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07493

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 3/00, 13/00, 15/00; A61K 37/48, 37/62, 37/50, 37/52
US CL :424/530; 530/381, 382
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/530; 530/381, 382

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Remington's Pharmaceutical Sciences

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,627,879 (Rose et al) 09 December 1986, col. 2, lines 29-66, col. 7, lines 15-39.	1-10, 52
Y	JP,A, 0,019,011 (Daiichi Pharm Co Ltd) 20 February 1974, abstract.	1, 11-12
Y	US, A, 4,175,182 (Schmer) 20 November 1979, abstract, col. 1, 59-68, col. 2, 1-14.	1, 11-17
Y, P	US, A, 5,094,960 (Bonomo) 10 March 1992, col. 1, lines 16-28, col. 2, lines 9-21, col. 4, lines 49-68, col. 5, lines 1-2.	18-40
Y	US, A, 4,362,567 (Schwarz et al) 07 December 1982, col. 2, lines 28-63.	1-10, 41-46, 50-56
Y	US, A, 4,427,650 (Stroetmann) 24 January 1984, col. 5, lines 20-45, col. 6, lines 48-49, col. 8, lines 21-38.	1-10, 41-46, 52-56, 60-68
Y	Remington's Pharmaceutical Sciences, Fifteenth edition, published 1975, Chapter 67, page 1252.	24, 26, 47-49, 57-59
Y	US, A, 4,442,655 (Stroetmann) 17 April 1984, col. 5, lines 8-36.	52-53, 60-68

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

23 December 1992

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07493

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US, A, 5,138,034 (Uemura et al) 11 August 1992, col. 2, lines 6-26, 45-68, col. 3, lines 1-35, col. 9, lines 61-68, col. 10, lines 1-2.	1-2, 11-12, 18-40
Y	Thrombosis Research, Volume 1, issued 1972, Michio Matsuda, et al, "A Simple, Large Scale Method For Preparation Of Plasminogen-Free Fibrinogen", pages 619-630, especially pages 620 and 622.	1, 11-12